International Journal of Current Research and Review
DOI: 10.31782/IJCRR.2018.10107

Research Article

IJCRR
Section: Life sciences
Sci. Journal Impact Factor: 5.385 (2017)
ICV: 71.54 (2015)

Effect of *Terminalia chebula* and Gallic Acid on Increased Adiposity of High-Fat Diet Induced Hyperlipidemic Mice

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ABSTRACT

Objective: To elucidate the role of *Terminalia chebula* and Gallic Acid on increased adiposity and as a regulator of Lipogenesis and its associated factors in high-fat diet induced hyperlipidemic mice.

Methods: The study was performed on C57BL/6J Male mice aged 6-8 weeks. Control was fed with normal diet and the other 3 groups were fed with commercial hyperlipidemic diet. Adipose tissue markers Peroxisome proliferator-activated receptor-gamma, CCAAT/enhancer-binding proteins-beta, 4-Hydroxy-2-nonenal, Thioredoxin, Tumour necrosis factor-α, adiponectin and leptin were estimated using adipose tissue by ELISA.

Results: The diet-induced hyperlipidemic C57BL/6J Male mice showed a marked reduction in the levels of transcription factors, oxidative stress markers and pro-inflammatory markers when treated with *Terminalia chebula* (Peroxisome proliferator-activated receptor-gamma (P<0.001),CCAAT/enhancer-bindingproteins-beta(P<0.001),4-Hydroxy-2-nonenol(P<0.001), Thioredoxin (P<0.01) and Tumour necrosis factor-α (P<0.001) which was more effective than Gallic Acid(Peroxisome proliferator-activated receptor-gamma(0.05),CCAAT/enhancer-binding proteins-beta(P<0.01), 4-Hydroxy-2-nonenal (P<0.01), Thioredoxin(P<0.05) and Tumour necrosis factor-α (P<0.01) and an elevation in adipokines- adiponectin and leptin in *Terminalia chebula* treated group- adiponectin (P<0.001)and leptin (P<0.001) was seen which was more significant than Gallic Acid- adiponectin (P<0.01) and leptin (0.05) when compared to group II HFD.

Conclusion: The study indicates the efficacy of *Terminalia chebula* as a potent antihyperlipidemic herbal drug which helps in controlling the increased adiposity by regulating the key markers in adipose tissue.

Key Words: *Terminalia chebula*, Hyperlipidemia, Adiposity, Gallic Acid

INTRODUCTION

Hyperlipidemia is a metabolic syndrome which includes lipid abnormalities (1,2). Regular intake of high-fat diet can result in hyperlipidemia (3). Hyperlipidemia is associated with an increase in the number of adipocytes due to enhanced adipogenesis. Adipose tissue is an important organ involved in dynamic regulation of metabolism in living body (4), our present study monitored the biochemical changes in adipose tissue by examining the expression of 4-Hydroxy-2-nonenal, Thioredoxin, adipogenic transcriptional factors, and adipokines. The important transcriptional factors Peroxisome proliferator-activated receptor-gamma and CCAAT/enhancer-binding proteins-beta required for adipogenesis and lipid metabolism are up-regulated in individuals whose diet composed of fatty foods (5,6) demanding the need for their screening during Hyperlipidemia. 4-Hydroxy-2-nonenal (HNE) an, α β-unsaturated aldehyde generated due to the lipid peroxidation (7) may alter the expression of antioxidants. One of the antioxidant playing a vital role in regulating redox status is Thioredoxin (8). Adipokines are peptides produced by adipose tissue involved in the regulation of glucose and lipid metabolism (9). Since adipokines were bound to fluctuate in the hyperlipidemic condition associated with adiposity (10,11), the expression of pro-inflammatory adipokine such as leptin, tumor necrosis factor-alpha, and anti-inflammatory adipokine- adiponectin was observed in

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ISSN: 2231-2196 (Print) **ISSN:** 0975-5241 (Online)
Received: 24.04.2018 **Revised:** 03.05.2018 **Accepted:** 14.05.2018

adipose tissue

In the present study, Gallic acid and were used for assessing their effect during hyperlipidemia. Gallic acid is 3, 4, 5, - trihydroxybenzoic acid, used for the treatment of a vast number of diseases such as cancer, cardiovascular diseases, ulcers, arthritis, gout etc. It is found to be antihyperglycemic, antihyperlipidemic, antioxidant, and cardioprotective (12). is a common herbaceous plant used in ayurvedic preparations as it possesses diverse medicinal properties which include antimicrobial, antioxidant, antiviral, anticarcinogenic, antihyperlipidemic, etc. Ellagic acid, gallic acid, chebulinic acid, chebugalic acid and corilagin present in this fruit have a beneficial effect in the treatment of hyperlipidemia (13,14).

MATERIALS AND METHODS

Preparation of T. chebula aqueous powder :

(Haritaki) dried fruits were collected from the Deciduous forest area in Kombankaddu area (Kodaikanal to Palani route).It was authenticated at CAPTAIN SRINIVASA MURTI RESEARCH INSTITUTE OF AYURVEDA AND SIDDHA DRUG DEVELOPMENT, Arignar Anna Government Hospital Campus, Arunbakkam, Chennai, Tamil Nadu. Seeds from the individual fruit were removed, washed thoroughly and then air-dried on a drier table at room temperature. Then the dried pulp crushed in an electrical mixer-grinder into a coarse powder. The powder was stored in a closed vessel for future use.

Animals:

Mice –C57BL/6J Male aged 6-8 weeks obtained from CPCSEA approved Breeder were taken for this experiment and kept at Centre for Toxicology and Developmental Research (CEFT) Sri Ramachandra University (SRU). All animal experiments were conducted as per the instructions of Institutional Animal Ethics Committee (IAEC/XLVIII/SRU/495/2016).

Experimental Design:

Grouping:

Animals were randomised and grouped based on the stratified body weight.

Group 1: Normal Control	- 10 nos
Group 2: Hyperlipidemic control	- 10 nos
Group 3: Hyperlipidemic mice given gallic acid orally	-10 nos
Group 4: Hyperlipidemic mice given orally	-10 nos

Induction of hyperlipidemia

Hyperlipidemia was induced to all the animals (GII – GIV) by giving commercially procured High fat diet (HFD) for a period of 15 weeks except Group I. Group I animals received standard rodent pellet diet for 15 weeks.

Solubility / Suspendability, Stability, and Homogeneity of Formulation

Solubility / Suspendability of the test and reference item (Gallic acid and Terminalia chebula) were ensured one day before dosing. 0.5% CMC Vehicle was selected based on the nature of test and reference drug. 0.5% CMC as a vehicle used for the preparation of test and reference drug. The homogeneity of test item in 0.5 % CMC was ensured using glass rod while dosing.

Histopathology

Liver, adipose were collected from all animals of the all dose groups and preserved in 10% neutral buffered formalin. The fixed tissues were processed, embedded in paraffin wax, sectioned approximately at 3-5 microns thick and stained with Haematoxylin and eosin for histopathological examination.

ELISA Studies

200 mg of adipose tissue was homogenized in standard homogenization buffer with anti-proteases (20 mM Tris-HCl, 1 mM EDTA, 255 mM sucrose, pH 7.4, anti-protease). The homogenates were centrifuged at 1,000 g for 10 min and the subnatant (whole tissue extract) below the lipid cake was aspirated and denatured by adding SDS to a final concentration of 0.06% and boiling for 10 min at 100°C. Total protein concentrations of the tissue extracts were measured using the method of Bradford (1976). Based on the requirement, the sample was diluted to acquire 100 μg of protein by using PBS buffer and used for the ELISA.

- PPAR-γ was quantified using ELISA kit (MBS005886, MyBioSource, USA)
- C/EBP-β was quantified using ELISA kit (MBS006925, MyBioSource, USA)
- Leptin was quantified using Leptin ELISA kit (11-LEPHU-E01, ALPCO, India)
- Adiponectin was quantified using Total Adiponectin ELISA kit (47-ADPHUT-E01, ALPCO, India)
- Tumour necrosis factor-α was quantified using ELISA kit (K0331131, Koma Biotech, Korea)
- 4-Hydroxy-2-nonenal was quantified using 4 hydroxy nonenol ELISA kit (MBS161454 96T, My Biosource, USA)
- Thioredoxin was quantified using Trx ELISA kit (MBS009625, MyBioSource, USA).

Statistical Analysis

The results were expressed as mean value standard deviation. Statistical analysis of the data was carried out using SPSS software. Statistical significance was arrived by comparing

results of One Way ANOVA of Group I & II with III & IV.

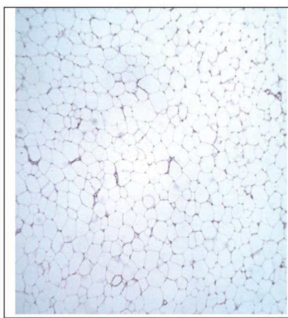
RESULTS

Histopathological Findings

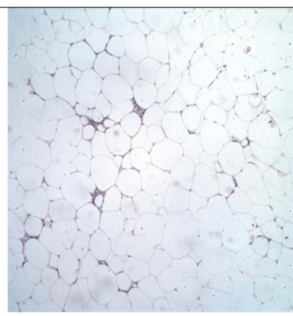
Histopathology of adipose tissue of Group I (Control) showed the normal size of adipose cells. However, the adipose of Group II (HFD) showed marked hypertrophy of adipose cells with a minimal degree of severity of inflammatory cell infiltration in crown shaped structure between adipose cells. In Group III (HFD + Gallic acid treatment group) Histopathology of adipose tissue revealed decrease in the size of adipose cells and no infiltration in crown shaped structure when compared to high-fat diet (G2) group animals and Group IV (HFD +   treatment group) revealed normalization of adipose cells when compared with normal control group animals.

The remarkable decrease in severity and incidence of lesions in adipose tissues of animals was observed in Group 4 animals as compared to the high-fat diet (G2) and gallic acid (G3) treated animals.

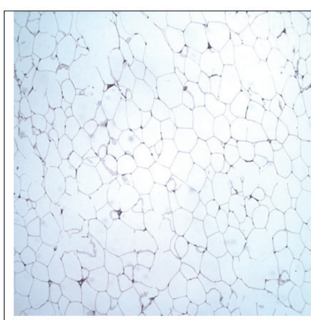
Transcription Factors in Adipose



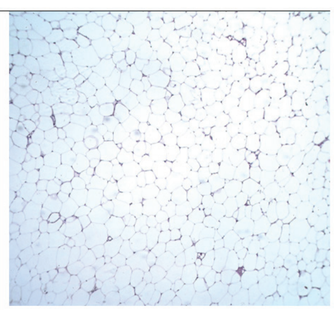
Group 1: Photomicrograph of adipose tissue showing normal size of adipose cells (H&E, X100)



Group 2: Photomicrograph of adipose tissue showing marked hypertrophy of adipose cells with minimal degree of inflammatory cell infiltration in crown shaped structure (black arrow) between adipose cells (H&E, X100)



Group 3 : Photomicrograph of adipose tissue showing decrease in the size of adipose cells and no infiltration in crown shaped structure (H&E, X100)



Group 4 : Photomicrograph of adipose tissue showing normal size of adipose cells (H&E, X100)

From the ELISA studies conducted on the adipocytes of all the 4 groups the levels of Peroxisome proliferator-activated receptor-gamma & CCAAT/enhancer-binding proteins-beta were found to be significantly increased in Group compared to Group I. The levels of Peroxisome proliferator-activated receptor-gamma and CCAAT/enhancer-binding proteins-beta were found to decrease significantly compared to HFD in Group IV (HFD +) than the Group III (HFD + Gallic Acid)(Table I).

Adipokine Levels

From the Elisa studies conducted on the adipocytes of all groups, the levels of Adiponectin and Leptin were found to be normal in Group I which was remarkably reduced in Group II (HFD). On receiving treatment the levels of Adiponectin and Leptin were found to increase considerably compared to HFD in Group IV (HFD +) than the Group III (HFD + Gallic Acid). However the levels of Tumour necrosis Factor -α which was found to be less in Group I (Normal) and increased in Group II (HFD) decreased remarkably in Group IV (HFD +   ) than the Group III (HFD + Gallic Acid) as compared to HFD. (Table II)

Oxidative stress in Adipose Tissue

The ELISA studies conducted on the adipocytes of 4 groups revealed normal levels of 4-Hydroxy-2-nonenal and Thioredoxin in Group I (Normal) which was notably increased in Group II (HFD). On treatment, the levels of 4-Hydroxy-2-nonenal and Thioredoxin were found to decrease appreciably in Group IV (HFD +) than the Group III (HFD + Gallic Acid)(Table III).

DISCUSSION

Adipose tissue is an important organ where excess fat in our body is stored. It is a dynamic organ that plays an important role in energy balance and homeostasis of our body. It acts as a sensor for lipid levels in our body and controls hunger, satiety and sleep patterns. Adipocytes are considered a major endocrine organ as they secrete lipid and protein factors which produce an impact on the metabolism of other tissues, regulation of appetite, immunological responses and vascular disease etc (15). The development of hyperlipidemia as a result of High fat diets (HFD) lead to an expansion of adipose tissue along with increase in size and number. The Adipose tissue inflammation is an important marker for hyperlipidemia. Our study observed changes in the structure of adipocyte in group II (HFD) mice which shows marked hypertrophy of adipose cells with minimal degree of inflammatory cell infiltration in crown shaped structure in the his-

topathological report of the adipose tissue .Herbal treatment by has resulted in decrease in hypertrophy of adipose tissue as assimilation of fat is decreased in the body The results are more predominant than Group III –Treatment with pure compound Gallic as is rich in fibre which reduces lipid absorption in the small intestine. Adipose tissue is composed of more of fatty acids and phospholipids and less of triglyceride in the form of neural fat. The presence of redox systems in microsomes in the adipose tissue results in high activity of lipid peroxidation (16). Oxidative stress and inflammation go hand-in-hand in the many tissues that are affected because oxidative stress induces the production of inflammatory cytokines, and the cytokines in turn induce free radical production. Lipid peroxidation (LP), an autocatalytic process is initiated by free radical attack on the unsaturated bonds of membrane fatty acids which leads to 4-Hydroxy-2-nonenal . In our study the levels of 4-Hydroxy-2-nonenal were found to increase significantly in Group II (HFD) as compared to Group I (Normal diet) as a result of high incidence of lipid peroxidation due to hyperlipidemia. The increase in HNE levels indicates damage to adipose tissue which is confirmed by the histopathological report(17).The levels of 4-Hydroxy-2-nonenal were found to be reduced in group IV (HFD+) as inflammation and oxidative stress is found reduced by the herbal drug.(Table III) The results are far more appreciable than treatment with Gallic acid as is a better drug in controlling fat assimilation in the adipose (evident from histopathological reports& Fig 6). Thioredoxin (TRX) is a redox protein that is involved in many biological functions which controls a number of transcription factors (18). Increased lipid peroxidation associated with increase in the levels of LDL results in the formation of oxLDL. This oxLDL further aggravates the problem by increasing its uptake by macrophages in adipocytes. This increases Thioredoxin expression and so the levels of Thioredoxin were found to be increased by the consumption of high fat diet as compared to mice that was given normal diet (Table III). The treatment by the herbal drug reduced the levels of LDL and lipid peroxidation causing decrease in the levels of Thioredoxin and this effect was more pronounced than treatment with Gallic Acid ,pure compound suggesting that is a better drug in treating oxidative stress and its associated complications caused by hyperlipidemia (19)(Table III & Fig 7). Further increase in the levels of lipids leads to increased Adipogenesis. Fat cell formation or adipogenesis is a process characterised by changes in factors that determine the structure of adipocyte(20). It is a process in which preadipocytes develop into mature adipocytes .This is a multistep process controlled by various transcriptional factors (15). The two factors that have a pronounced effect on this synthesis are Peroxisome proliferator-activated receptor-gamma and CCAAT/enhancer-binding proteins-beta (21). CCAAT/enhancer-binding proteins-beta genes are rapidly induced to express which then activate Peroxisome proliferator-activated receptor-gamma (22). Peroxisome proliferator-activated receptor-gamma can initiate adipogenesis program, giving rise to fat cells by activating the adipogenic genes responsible for adipogenesis (23). In our study increased levels of both Peroxisome proliferator-activated receptor-gamma and CCAAT/enhancer-binding proteins-beta were observed in group II(HFD) compared to group I (Normal diet) which has lead to increased adipogenesis evident from the histopathological studies which indicate increase in fat depots of adipose tissue (Table I &Fig 1 & 2) . The adipokines which keep the adipogenesis at check by reducing lipogenesis and favouring lipolysis are leptin and adiponectin which are drastically reduced in hyperlipidemia (24)(Table II). The deficiency of leptin or leptin receptor is seen in animals fed with high fat diet which leads to extreme obesity(25)(Fig 3). Adiponectin is a peptic hormone which decreases serum FFA, glucose, and triacylglycerol concentrations in the adipose tissue (26). However adiponectin concentrations fall with increasing obesity which was observed in group II (HFD) mice(Table II, Fig 4) which indicated both decreased adiponectin and leptin levels .The herbal drug is more efficient than Gallic Acid in increasing the levels of both adiponectin and leptin which is evident from their levels in group III(HFD+GA) and group IV (HFD+TC) and thereby decreasing adipogenesity.(Fig 3 & 4)However, the Tumor necrosis factor-alpha (TNF alpha) a multifunctional cytokine which exerts a series of biological actions on adipose and regulates or interfere with adipocyte metabolism by regulating fatty acid metabolism (27) is downregulated more effectively by Terminalia chebula than pure compound Gallic Acid.(Table II & Fig 5) . Our study indicates that herbal treatment with brings about a reduction in fat content of adipose as it reduces adipogenesis by downregulating the transcription factors, Tumour necrosis factor α, and oxidative stress markers and brings about lipid breakdown or lipolysis by increased production of adiponectin and leptin more effective than pure compound Gallic Acid.

CONCLUSION

These results suggest that could decrease adipose tissue mass by down-regulation of transcriptional factors- Peroxisome proliferator-activated receptor-gamma, CCAAT/enhancer-binding proteins-beta, Pro-inflammatory cytokine-TNF α, Oxidative stress markers – 4-Hydroxy-2-nonenal, Thioredoxin and upregulation of adipokines that favor lipolysis – adiponectin and leptin. This effect has been far more pronounced than group III mice treated with Pure compound Gallic Acid (50 mg/Kg body wt)). Further, to confirm the anti-adipogenic activity of more effective than Gallic Acid by histopathological examination of adipose tissue. The following results prove a more potent antihyperlipidemic factor than pure compound Gallic Acid.

ACKNOWLEDGEMENT

The authors acknowledge the immense help received from the scholars whose article cited and included in references of this manuscript. The authors are also grateful to authors/editors/publishers of all those articles, journals and books from where the literature for this article has been reviewed and discussed. Authors acknowledge the immense help received from the scholars whose articles are cited and included in references of this manuscript. The authors are also grateful to authors / editors / publishers of all those articles, journals and books from where the literature for this article has been reviewed and discussed.

Conflict of interest: nil

Financial Support: nil

Ethical Clearance: (IAEC/XLVIII/SRU/495/2016)

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Table I: Levels of Transcription Factors in Adipose tissue in diferent groups

Parameters	Normal Control	Hyperlipidemic Control	Gallic acid 50 mg/kg	T. Chebula 250mg/kg
Peroxisome proliferator-activated receptor-gamma	2.997±0.27	9.2351±0.075 ***	8.157±0.53 #	5.419±0.52 ###
CCAAT/enhancer-binding proteins-beta	0.025±0.001	0.0488±0.003 8***	0.035±0.004 ##	0.0318±0.004 ###

* Significant compared to the normal control

Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10,

- *P<0.05, ## - **P<0.01, ### - ***P<0.001

Table II: Levels of Adipokines in Adipose tissue of different groups

Parameters	Normal Control	Hyperlipidemic Control	Gallic acid 50 mg/kg	T. Chebula 250mg/kg
Leptin (ng/mg protein)	3.1± 0.27	2.52±0.20*	2.94±0.17#	3.44±0.29###
Adiponectin (ng/mg protein)	6.04±0.43	3.4±0.41***	4.8±0.32##	5.26±0.35###
Tumour necrosis factor α (pg/mg protein)	2.34±0.15	3.68±0.26***	2.5±0.22##	2.46±0.19###

* Significant compared to the normal control

Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10,

- *P<0.05, ## - **P<0.01, ### - ***P<0.001

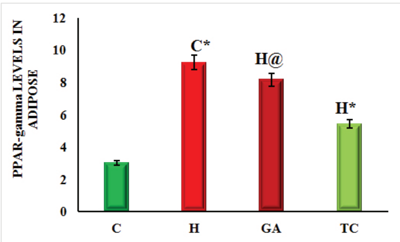
Table III: Levels of Oxidative stress markers in Adipose tissue of different groups

Parameters	Normal Control	Hyperlipidemic Control	Gallic acid 50 mg/kg	T. Chebula 250mg/kg
4-Hydroxy-2-nonenal (ng/mg protein)	7.38±0.71	13.78±0.93***	9.14±0.82##	8.62±0.81###
Thioredoxin (ng/mg protein)	9.22±0.79	10.76±1.12*	8.78±0.76#	8.46±0.77##

* Significant compared to the normal control

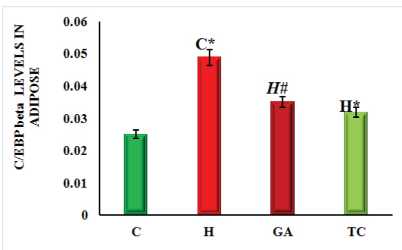
Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10,

- *P<0.05, ## - **P<0.01, ### - ***P<0.001



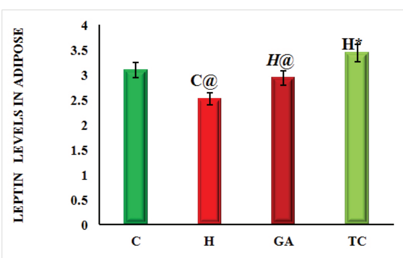
C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

Figure 1: Peroxisome proliferator-activated receptor-gamma Levels in Adipose Tissue of Different Groups.



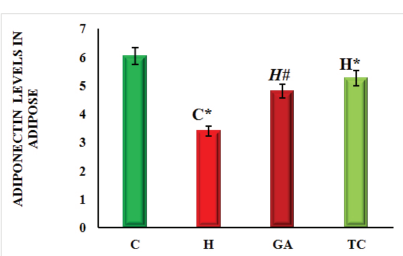
C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

Figure 2: CCAAT/enhancer-binding proteins-beta Levels in Adipose Tissue of Different Groups.



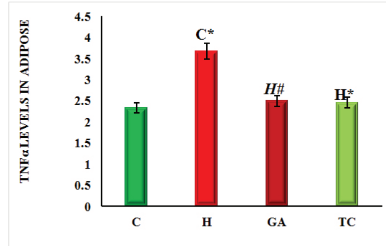
C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

Figure 3: Leptin Levels in Adipose Tissue of Different Groups.



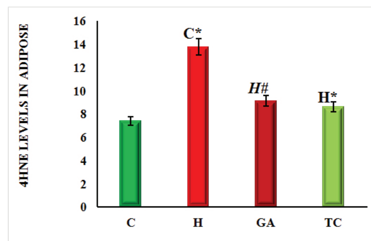
C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

Figure 4: Adiponectin Levels in Adipose Tissue of Different Groups.



C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

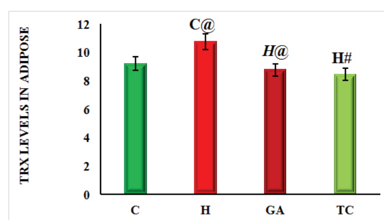
Figure 5: Tumour necrosis factor α (TNFα) Levels in Adipose Tissue of Different Groups.



C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

Figure 6: 4-Hydroxy-2-nonenal(4HNE) Levels in Adipose Tissue of Different Groups.

Thioredoxin (TRX) LEVELS IN ADIPOSE TISSUE OF DIFFERENT GROUPS



C- Significant compared to the normal control
H- Significant compared to the Hyperlipidemic control (One-way ANOVA test, n=10),
@ -P<0.05, #- P<0.01, * -P<0.001

Figure 7: Thioredoxin (TRX) Levels in Adipose Tissue of Different Groups.